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Experimental Co-Expression of Vimentin and Keratin Intermediate Filaments in Human Breast Cancer Cells Results in Phenotypic Interconversion and Increased Invasive Behavior

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The expression of intermediate filament proteins is remarkably tissue specific, which suggests that the intermediate filament type(s) present in cells is somehow related to their biological function. However, in some cancers, particularly malignant breast carcinoma, there is a strong indication that vimentin is co-expressed with keratins, thus presenting as a dedifferentiated or interconverted (between epithelial and mesenchymal) phenotype. In the present study, we recapitulated the interconverted phenotype by developing stable transfectants of MCF-7 human breast cancer cells, termed MoVi clones, to express both vimentin and keratins. Overexpression of vimentin in these cells led to augmentation of motility and invasiveness in vitro. These activities could be transiently down-regulated by vimentin antisense oligonucleotides in MoVi clones and MDA-MB-231 cells (which constitutively co-express keratins and vimentin). Furthermore, in the MoVi experimental transfectants expressing the highest percentage of vimentin-positive cells, their proliferative capacity, clonogenic potential, and tumorigenicity increased. However, the metastatic ability of the MoVi transfectants remained unchanged compared with MCF-7neo controls. The MDA-MB-231 cells metastasized to axillary lymph nodes in a SCID mouse model. Finally, we explored the possibility that potential changes could occur with respect to cell surface integrins. These studies revealed a decrease in the α_2- and α_3-containing promiscuous integrins, in addition to β_1 containing integrins, concomitant with an increase in the α_6-containing laminin receptor integrin. Further functional analysis of the α_6 observation showed an increase in the haptotactic migration of MoVi transfectants toward a laminin substrate. From these data, it is postulated that the ability to co-express vimentin and keratins confers a selective advantage to breast cancer cells in their interpretation of signaling cues from the extracellular matrix; however the addition of vimentin intermediate filaments alone is not sufficient to confer the metastatic phenotype. (Am J Pathol 1997, 150:483–495)

One of the major problems with respect to the pathophysiology of cancer is, undeniably, metastasis. This intricate, multistep biological process consists of the recurrent migration and invasion of cancer cells in the vasculature and tissue parenchyma, which perpetuates tumors at multiple locations throughout the body. Of the many types of cancers facing diagnosis and treatment, breast carcinoma has been increasing at an alarming rate in the United States over the past two decades.[1] Although the etiological agents of this disease remain enigmatic, some strides have been made with respect to the identification of diagnostic markers associated with benign *versus* malignant disease. However, relatively little remains known about the fundamental biology of these markers involved in the pathological progression of breast cancer.

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From a clinical point of view, early detection of breast carcinoma determines disease prognosis. In this regard, one of the classical diagnostic markers of this disease has been cytokeratins, indicative of epithelial cells and a member of the intermediate filament (IF) family of proteins.[2] However, recent reports have indicated that some metastatic human breast cancers (examined both *in situ* and *in vitro*) can co-express an additional IF marker, vimentin, indicative of mesenchymal lineage and correlated with increased invasive and metastatic potential.[3–6] Direct experimental evidence supporting this contention, however, is somewhat controversial.[7,8]

Indeed, IF biology represents an emerging and exciting field in cell biology, with significant applications in diagnostic pathology. The expression of IF proteins is remarkably tissue specific, which suggests that the IF type(s) present in cells is somehow related to their biological function.[9,10] Of the three major types of cytoskeletal proteins in eukaryotic cells, actin filaments, IFs, and microtubules, IFs are the most complex.[11–14] In fact, there are approximately 50 different IF proteins that can be further subdivided (according to the most recently published classification scheme) as follows: type I, acidic keratins; type II, neutral and basic keratins; type III, vimentin, desmin, peripherin, and glial fibrillary acidic protein; type IV, neurofilaments, nestin, and α-internexin; and type V, nuclear lamins.[9–14] One major difference associated with keratins that distinguishes them from other members of the IF family, such as vimentin, is that keratins are expressed as heteropolymer pairs consisting of specific type I and type II proteins. Their expression is highly regulated during embryonic development and cellular differentiation[15]; for example, keratins 8 and 18 are the first IF proteins to be expressed during embryogenesis.[16,17] Furthermore, co-expression of vimentin and keratins in parietal endoderm cells has been implicated in embryogenesis.[18] Interestingly, highly metastatic tumor cells have been compared with a dedifferentiated or interconverted (between epithelial and mesenchymal) phenotype,[19] with little information regarding the regulation and biological dynamics of this event. Although the exact function of IF proteins remains elusive, defects in the genes encoding these proteins have been shown to contribute to degenerative disease.[9,10,20] Moreover, a dynamic role has been suggested for IFs with respect to their involvement as signal transducers, relaying information from the extracellular matrix (ECM) to the nucleus.[13]

The present study has focused on recapitulating vimentin and keratin IF co-expression in human breast cancer cells in an *in vitro* model by developing stable experimental transfectants and comparing their biological properties with breast cancer cells that constitutively express both IFs. With these models, vimentin expression is up-regulated and down-regulated, and the consequence of these manipulations is examined with respect to changes in tumor cell migration, invasion, clonogenicity, and tumorigenesis. Our objective was to achieve a better understanding of the regulation of motility in breast cancer cells expressing vimentin and keratins and to test the hypothesis that an ability to co-express vimentin and keratin IFs confers a selective advantage to breast tumor cells in their interpretation of signaling cues from the ECM.

Materials and Methods

Cell Culture

MCF-7, a human breast cancer cell line of poor invasive and metastatic potential, was kindly supplied by Dr. Fred Miller (Michigan Cancer Foundation, Detroit, MI). The MDA-MB-231 human breast cancer cell line, of high invasive and metastatic potential, was originally obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 10 mmol/L HEPES (pH 7.3), and 10 mmol/L L-glutamine. Cell proliferation and doubling times were determined by plotting standard growth curves with cells harvested and counted by hemocytometer at 48-hour time points. Cell cultures were determined to be free of *Mycoplasma* contamination using the Gen-Probe rapid detection system (Fisher, Pittsburgh, PA).

Expression Vector Construction

For expression of vimentin, a 1.8-kb *Bam*HI cDNA insert, encoding the full-length mouse vimentin protein,[21] was subcloned into the *Bam*HI site of the vector LK444.[22] In this vector, the cDNA is expressed under the control of the human β-actin promoter.[23] In addition, LK444 encodes the neomycin phosphotransferase gene derived from pSV2neo, allowing for stable selection of transformants in the antibiotic G418 (Life Technologies).

DNA Transfection and Clone Selection

MCF-7 cells (2×10^5 cells in 6-cm dishes) were transfected with 10 μg of the LK444-mouse vimentin vector by calcium phosphate precipitation[24] in the presence of DMEM supplemented with 10% fetal bovine serum. After overnight incubation, the medium was replaced with fresh RPMI 1640 with 10% fetal bovine serum. The next day cells were plated at 1×10^5 cells in 10-cm dishes in media supplemented with 400 μg/ml G418-containing media. After 20 days, individual clones were isolated and analyzed for mouse vimentin content by indirect immunofluorescence. Stable subclones were maintained in media with 200 μg/ml G418.

Vimentin Antisense Knock-Out

To transiently diminish the expression of vimentin IFs, phosphorothioate-modified oligodeoxynucleotides (commercially prepared by Biopolymer Labs, Camden, NJ) were used in the sense and antisense orientation: sense sequence (5′ to 3′), GCCATGTCCACCAGGTCC (18-mer); antisense sequence (5′ to 3′), GGACCTGGTGGACATGGC (18-mer). MCF-7 and MDA-MB-231 human breast carcinoma cells were seeded overnight into a six-well dish (50,000 cells/well in 2 ml of DMEM/10% fetal bovine serum). The cells were washed once with serum-free Opti-MEM I medium (Life Technologies) and then incubated for 4 hours at 37°C with 1 μmol/L sense or antisense modified oligodeoxynucleotides in a 10 μg/reaction lipofectamine delivery system (Life Technologies). The cells were then washed with Opti-MEM medium, harvested with 2 mmol/L EDTA, and resuspended in Opti-MEM/1X MITO+ (Collaborative Biomedical, Bedford, MA) for use in a transfilter migration assay. Briefly, 5×10^4 cells were seeded onto a gelatin-soaked polycarbonate membrane in a membrane invasion culture system (MICS) chamber[25] and incubated at 37°C for 5.5 hours. The migratory cells were removed from the lower wells and scored for their migratory ability as compared with an untreated control. Cells treated as above were also seeded onto coverslips coated with Cell-Tak (Collaborative Biomedical) to verify vimentin knock-out after 5.5 hours, using indirect immunofluorescence microscopy, as described below.

Immunohistochemical Analysis of Intermediate Filaments

Dual immunofluorescence labeling of keratins 8 and 18 and vimentin IFs was accomplished using antibody CK-5 (Sigma Chemical Co., St. Louis, MO) conjugated with fluorescein isothiocyanate for the keratins and V-9 (Dako, Carpinteria, CA) conjugated with Texas Red-X for vimentin. These antibodies were conjugated with their respective fluorochromes using the FluoReporter protein-labeling kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol after an initial precipitation and purification of these antibodies using protein-A-Sepharose (Pharmacia, Piscataway, NJ).

Cells were seeded onto glass coverslips to 70% confluence. Subsequently, cells were washed three times with room temperature phosphate-buffered saline (PBS) and then fixed in 100% cold methanol for 5 minutes. Coverslips were incubated with the cocktail of V-9 conjugated to Texas Red-X (for the detection of vimentin) and CK-5 conjugated to fluorescein (for the detection of keratins 8 and 18) for 1 hour, rinsed in PBS, and mounted onto a glass microscope slide using gelvatol. Controls for nonspecific staining consisted of omitting the primary antibody and treating the cells with secondary fluorescent label only. Positive controls consisted of indirect immunofluorescence labeling, using either V-9 or a rabbit polyclonal antibody recognizing mouse vimentin[21] or monoclonal antibody CK-5 recognizing human K18, followed by the appropriate fluorochrome-conjugated secondary antibody. All coverslips were observed with a Zeiss 410 LSM confocal microscope and photographs recorded with a Sony Mavigraph color printer. Quantification of vimentin IF fluorescence and percent positivity was performed by the KS-300 image analysis program in conjunction with the Zeiss 410 LSM confocal microscope.

Invasion Assay

Tumor cells (1×10^5) were seeded into the upper wells of the MICS chamber[25] onto laminin/collagen IV/gelatin-coated (Sigma) polycarbonate filters (containing 10-μm pores) in DMEM containing 10% NuSerum (Collaborative Biochemical). After 24 hours of incubation at 37°C, the cells that invaded the filter were collected, stained, and counted, as previously described.[25] Percent invasion was corrected for proliferation and calculated as follows:

$$\frac{\text{Total number of invading cells}}{\text{Total numer of cells seeded}} \times 100.$$

Migration Assay

Unstimulated motility was determined in MICS chambers containing polycarbonate filters (with 10-μm

pores) that had been soaked overnight in 0.1% gelatin, using a modification of a procedure described by McCarthy and colleagues.[26] Tumor cells (5×10^4) were seeded randomly in each upper well (six wells per cell line/experiment, performed in duplicate), allowed to incubate at 37°C for 5.5 hours in DMEM containing 10% NuSerum, and subsequently processed as described for the invasion assay.

Haptotatic motility was determined with respect to the ability of tumor cells to move toward a solid gradient of attraction (either gelatin or laminin), in the MICS chamber. The undersurfaces of polycarbonate filters were coated with a 1 mg/ml solution of human laminin (Sigma) or gelatin (control; Biorad, Hercules, CA) and processed as described above for determination of unstimulated motility.

Soft Agar Assay

Anchorage-independent growth analysis of human breast cancer cells was performed as previously described.[27] Each cell type was tested in triplicate for growth in soft agar. For determining the cloning efficiency in agar, 10^4 cells were plated in a 60-mm dish. Subsequently, liquid medium (0.5 ml) was layered over the agar cultures to prevent desiccation. The number of large colonies formed with dense centers (>0.1 mm in diameter) was counted per 10 random fields and averaged for each plate after 3 weeks.

Tumorigenicity Assay

To measure tumor formation *in vivo*, cells were harvested, washed with PBS, and resuspended in cold, serum-free, Hank's balanced salt solution (Life Technologies). Female scid/scid cb.17 mice were implanted orthotopically by subcutaneous injection into the mammary pad at 4 to 6 weeks of age (5×10^6 cells/injection). At 24 hours before the inoculation, mice received a subcutaneous estrogen (0.72 mg of 17β-estradiol) 60-day slow-release pellet (Innovative Research of America, Toledo, OH) interscapularly, *via* a 10-gauge trochar, as directed by the manufacturer's recommendations. Tumor formation was assessed once weekly and at the time of sacrifice (38 days).

Determination of Integrin Profiles

A total of 3×10^6 cells were harvested from the culture flasks using 2 mmol/L EDTA in PBS followed by a wash with serum-free RPMI medium containing 1% bovine serum albumin. The cells were then aliquotted into nine microfuge tubes in 0.5 ml of RPMI plus 1% bovine serum albumin, and 2 μg of monoclonal anti-integrin subunit antibodies (α_2, α_3, α_4, α_5, α_6, α_v, or β_1; Life Technologies) were then added: one antibody per tube with one tube receiving no antibody as a control for autofluorescence and another tube receiving only secondary antibody as a control for nonspecific binding of these cells by the secondary antibody. These samples were then incubated for 1 hour, washed twice with RPMI plus 1% bovine serum albumin, and then treated (except for the autofluorescence control sample) for 0.5 hour with an anti-mouse fluorescein-isothiocyanate-conjugated antibody (Fab'_2; Becton Dickinson). The cells were then washed as before, fixed in 3.7% formaldehyde in PBS for 10 minutes, washed again with PBS, and finally resuspended in 0.3 ml of PBS. These samples were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ), and the mean fluorescence levels were determined per treatment.

Results

Expression of Vimentin in Breast Cancer Cells

After the transfection of MCF-7 cells with the LK444-mouse vimentin vector or the LK444 vector containing the neomycin resistance marker only, G418-resistant clones were selected, expanded, and examined for the expression of vimentin as well as keratin 8 and 18 IFs (Figure 1). Dual immunofluorescence labeling of the parental MCF-7neo sham transfected cells revealed a well developed network of keratin 8 and 18 IFs without any vimentin. Similarly, dual labeling of MCF-7 parental cells showed positive staining for keratins 8 and 18 only (data not shown). Immunohistochemical analyses of three experimental clones expressing the mouse vimentin transgene demonstrate the degree of heterogeneity of vimentin IFs. All three clones (MoVi-3, MoVi-9, and MoVi-10) were positive for keratin 8 and 18 IFs. However, further quantification of vimentin IF positivity revealed that the MoVi-3 cells contained 47% vimentin IF-positive cells; MoVi-9 demonstrated approximately 55% vimentin-positive IFs; and MoVi-10 showed the lowest percentage of vimentin IF positivity, approximately 38%. Further cloning selection of the MoVi-10 cells resulted in the MoVi-10′ cells, which contained 65% vimentin-positive cells. By comparison, the highly invasive and metastatic MDA-MB-231 cells were 100% positive for the constitutive expression of both vimentin and keratin 8 and 18 IFs.

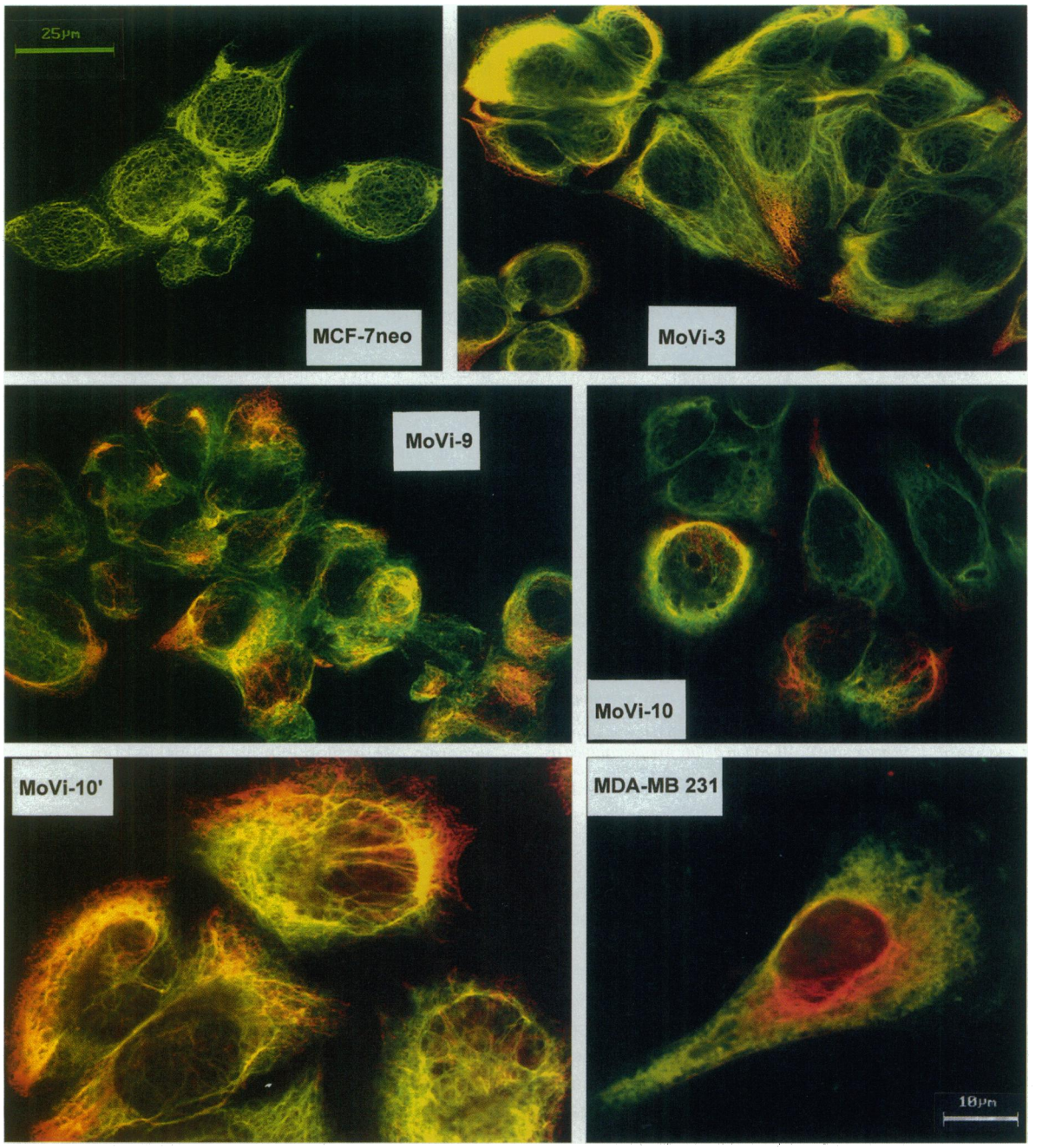

Figure 1. *Dual immunofluorescence labeling of keratins 8 and 18 (fluorescein) and vimentin (Texas Red) IFs in human breast cancer cells. Keratins 8 and 18 IFs are seen in only the MCF-7neo sham transfectants; keratins 8 and 18 and vimentin IFs are observed in the experimental transfectants MoVi-3, MoVi-9, MoVi-10, and vimentin enriched MoVi-10′ and also in the highly invasive and metastatic MDA-MB-231 cells, which constitutively express both IFs. Magnification, ×600 (MCF-7neo, MoVi-3, MoVi-9, and MoVi-10) and ×1180 (MoVi-10′ and MDA-MB-231).*

With respect to the morphological localization of vimentin IFs in the different experimental clones tested, a distinctive pattern emerged. MoVi-3 cells displayed vimentin IFs primarily within the extended pseudopodial processes, MoVi-9 cells contained vimentin IFs predominantly in the nuclear cage and perinuclear region, MoVi-10′ cells showed an extensive IF network within the pseudopodial processes and the peripheral regions of the cells, and MDA-MB-231 cells contained vimentin IFs in the perinuclear and nuclear cage region.

Invasive Potential of Breast Cancer Cells

Evidence from our laboratory and others indicates a strong correlation between keratin and vimentin co-

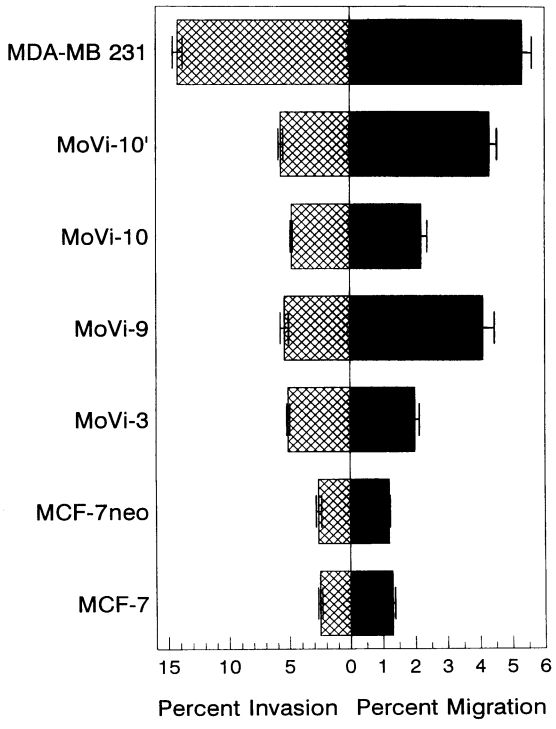

Figure 2. *Invasive and migratory ability of human breast cancer cells, as measured using a modified Boyden chamber (membrane invasion culture system).*[25] *Invasive ability was calculated as the percentage of cells capable of invading a laminin/collagen IV/gelatin-coated polycarbonate membrane over 24 hours, compared with the total number of cells seeded (± SE determined). Migration ability was determined as the percentage of cells harvested that migrated through gelatin-coated polycarbonate membranes over 5.5 hours, compared with the total number of cells seeded (± SE calculated).*

expression in human breast cancers and invasive and metastatic disease.[3-6] After generating stable experimental transfectants expressing the vimentin transgene (shown in Figure 1), we explored the effects of vimentin and keratin co-expression with respect to the invasive ability of these cells in an *in vitro* model (Figure 2). After 24 hours of incubation on a polycarbonate filter coated with a laminin/collagen IV/gelatin matrix situated within an invasion chamber, the invasive abilities of the vimentin and keratin IF-positive MoVi-3, MoVi-9, MoVi-10, and MoVi-10′ experimental transfectants were significantly greater than the keratin-positive MCF-7 and sham transfected MCF-7neo cells. By comparison, the MDA-MB-231 breast cancer cells, which constitutively express vimentin with keratins 8 and 18, showed the greatest ability to invade *in vitro* (14.5 ± 0.4%). Similar observations were made with regard to increased migratory ability through gelatin-coated filters. Thus, these data indicate that up-regulation of vimentin IF expression in keratin-positive breast cancer cells appears to be correlated and associated with increased invasive and migratory ability *in vitro*.

The next set of experiments focused on down-regulating vimentin expression in the highly invasive MDA-MB-231 cells and then examining the consequence of this action on the migratory ability of these cells. To execute this strategy, we treated the MDA-MB-231 (keratin and vimentin IF-containing cells), the MoVi-10′ transfectants, and the MCF-7 (keratin-only-containing cells) with 18-mer oligonucleotides to vimentin, in the sense and antisense orientation, and subsequently tested the ability of the transiently affected cells to migrate through gelatin-coated polycarbonate filters in the MICS chamber. Verification of the transient down-regulation of vimentin expression in MDA-MB-231 cells removed from suspension is shown in Figure 3A. Vimentin expression in the MoVi-10′ cells was also down-regulated (data not shown). The function-related results showed no effect on the migratory behavior of MCF-7 cells treated with either sense or antisense oligonucleotides (Figure 3B). However, there was a 70% reduction in the migration of MDA-MB-231 cells and a 50% reduction in migratory ability of the MoVi-10′ cells treated with antisense oligonucleotides to vimentin, with no significant change in associated controls' migratory behavior, either untreated or incubated with sense oligonucleotides. Hence, these data demonstrate that experimental down-regulation of vimentin expression in highly invasive human breast cancer cells results in a significant decrease in migratory ability, a key step in metastasis.

Growth Rate, Clonogenicity, and Tumorigenicity of Breast Cancer Cells

Although the data are not presented, measurements of growth rate clearly indicated that the doubling time (hours) for MoVi-10′ and MDA-MB-231 cells (range, 24 to 29 hours) were significantly less than for the MCF-7neo, MoVi-3, and MoVi-9 cells (range, 42 to 45 hours). The clonogenicity data demonstrated comparable numbers of colonies formed in soft agar by MCF-7neo and MoVi-3 cells (2.35 to 2.41) and MoVi-9 and MoVi-10′ cells (5.07 to 6.20) compared with MDA-MB-231 cells (14.03) (data not shown).

To assess potential differences in tumorigenicity, we orthotopically implanted each breast cancer cell line into the mammary pad of female scid/scid cb.17 mice and measured tumor formation over a 38-day period. These data indicate no significant difference in tumor size at the site of primary tumor inoculation in MCF-7neo *versus* MoVi-3 and MCF-7neo *versus* MoVi-9, as shown in Table 1. However, in the MoVi-10′ cells, significantly larger tumors were formed, as compared with

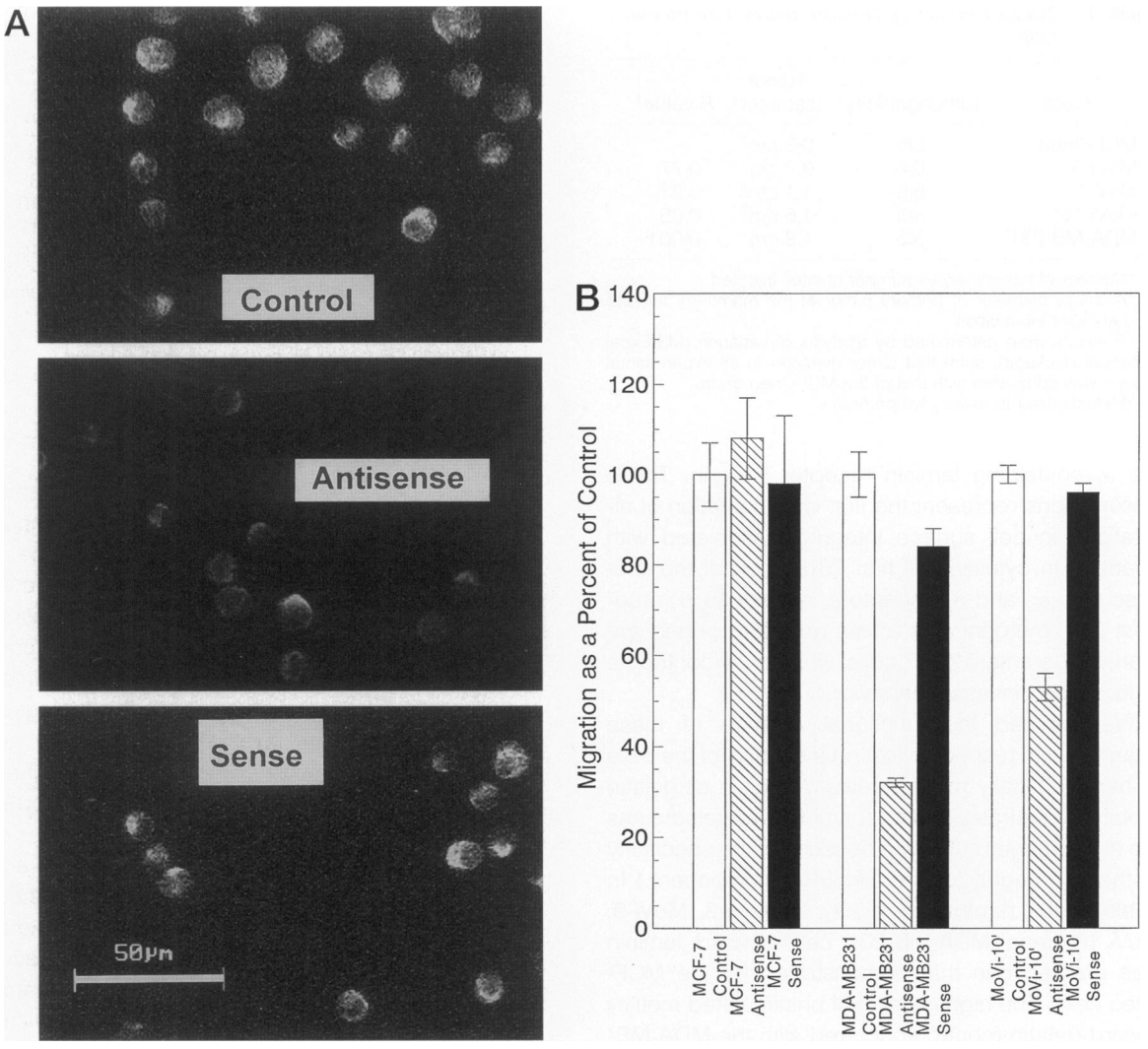

Figure 3. A: *Immunofluorescence staining of vimentin IFs in MDA-MB-231 cells from suspension, which were either untreated (control), treated with antisense oligonucleotides to vimentin (antisense), or treated with vimentin oligonucleotides in the sense orientation (sense) for 5.5 hours. Magnification, ×460.* **B:** *Effect of vimentin oligonucleotides on human breast cell migration. Keratin-positive MCF-7 cells, keratin- plus vimentin-positive MDA-MB-231 cells and MoVi-10' transfectants were treated with vimentin oligonucleotides in the sense and antisense orientation or remained untreated for 5.5 hours as described in* **A**. *Subsequently, these cells were allowed to migrate through gelatin-coated polycarbonate membranes for 5.5 hours, and the percentage of cells that were able to migrate compared with the migratory value(s) of either the MCF-7 untreated control or the MDA-MB-231 untreated control (± SE) was determined.*

MoVi-3, MoVi-9, and the MCF-7neo cells. No metastases were observed in any of the mice receiving these cell lines. The MDA-MB-231 cells generated the largest tumors and metastasized to the axillary lymph nodes. Collectively, these results indicate that the human breast cancer cells that express the vimentin transgene were not able to metastasize, like the MDA-MB-231 cells, within 38 days.

Determination of Changes in Cell Surface Integrins

As there were differences among the cell lines tested in invasive potential, growth potential, and to some extent, tumorigenicity, we examined the possibility that potential changes could occur with respect to cell surface integrin expression as a consequence of breast cancer cells co-expressing vimentin and keratin 8 and 18 IFs. Flow cytometric analysis of integrins on the cells used in this study demonstrated changes in some integrins associated with the interconversion of the epithelial-to-mesenchymal phenotype (Table 2). When comparing the mean fluorescence of the experimental clones with that of the MCF-7 cells, the most noteworthy changes observed were a decrease in α_2- and α_3-containing promiscuous ECM receptor integrins in addition to the β_1-containing integrins, with a concomitant increase in

Table 1. *Tumorigenicity of Human Breast Carcinoma Cells*

Cells	Tumorigenicity*	Tumor diameter†	*P* value‡
MCF-7neo	5/5	0.8 cm^3	–
MoVi-3	6/6	0.7 cm^3	0.77
MoVi-9	6/6	1.1 cm^3	0.27
MoVi-10′	5/5	1.6 cm^3	0.05
MDA-MB-231§	5/5	1.9 cm^3	0.001

*Number of tumors *versus* number of sites injected.
†Average diameter of primary tumor at the mammary fat pad 38 days after inoculation.
‡*P* values were determined by analysis of variance (MS-Excel statistical package), such that tumor diameter in all experimental groups was compared with that of the MCF-7neo group.
§Metastasized to axillary lymph nodes.

Table 3. *Haptotactic Migration of Human Breast Carcinoma Cells*

Cell line	% migration	
	Gelatin	Laminin
MCF-7neo	100 ± 18	100 ± 2
MoVi-3	85 ± 9	113 ± 3
MoVi-9	98 ± 12	140 ± 15
MoVi-10′	71 ± 5	152 ± 17
MDA-MB-231	138 ± 6	373 ± 10

Migration is expressed as percentage of cells that migrated through 10-μm pores in a polycarbonate filter toward gelatin- or laminin-coated substrates over 5.5 hours. Percent migration associated with the control MCF-7neo cells was equated to 100% ± SEM, and values for all other cells were compared accordingly.

the α_6-containing laminin receptor integrin. These observations represent the first demonstration of alterations in cell surface integrins associated with changes in cytoskeletal IFs. Diminution of the promiscuous α_2 and α_3 receptors, as well as β_1, suggest the emergence of a less adhesive phenotype than the parental MCF-7 cells, which may contribute to increased invasive behavior.

We pursued the functional analysis of these changes with respect to testing the ability of the cells to haptotactically migrate toward laminin or gelatin (control) substrates *in vitro*. (Laminin was selected as the experimental substrate because of the specificity of the α_6 integrin toward this ECM component.) In Table 3, the haptotactic ability of MoVi-3, MoVi-9, MoVi-10′, and MDA-MB-231 cells toward laminin was greater than that demonstrated by the MCF-7neo cells. The highest level of unstimulated motility toward gelatin (control) occurred with the MDA-MB-231 cells.

Discussion

In previous reports regarding breast cancer, there is strong evidence correlating the co-expression of vimentin and keratins IFs with invasive and metastatic disease.[3–5] The emergence of this dedifferentiated, interconverted cellular phenotype has raised important questions about the biological significance of this observation, especially with respect to the conference of metastatic properties. In the present study, we attempted to recapitulate the interconverted breast cancer cell phenotype *in vitro*, which provides a model in which to study the biological functions of migration, invasion, clonogenicity, tumorigenesis, and associated cell surface integrins, all independently and collectively important properties in the metastatic cascade.[28–30]

Indeed, the underlying question in the design and performance of this study was to determine whether the inappropriate expression of vimentin IFs in keratin-positive human breast cancer cells was causative or associative with respect to the metastatic phenotype. To appropriately address this question, we employed two separate strategies, which consisted of 1) overexpressing vimentin IFs in poorly invasive and nonmetastatic MCF-7 cells and 2) down-regulating the expression of vimentin IFs in MoVi-transfected cells, in addition to the highly invasive and metastatic MDA-MB-231 cells. Although each model possessed inherent flaws, interesting biological observations were achieved that assist in the elucidation of

Table 2. *Changes in Integrins Determined by FAC Analysis*

Integrin subunit	Mean fluoresence*		MoVi-3		MoVi-9		MoVi-10′	
	MDA-MB-231	MCF-7neo	Mean fluoresence	*versus* MCF-7neo	Mean fluoresence	*versus* MCF-7neo	Mean fluoresence	*versus* MCF-7neo
α_2	236	345	140	↓(0.41×)†	120	↓(0.35×)	135	↓(0.39×)
α_3	1336	187	142	↓(0.76×)	89	↓(0.48×)	80	↓(0.43×)
α_6	80	5	17	↑(3.40×)	15	↑(3.00×)	10	↑(2.00×)
β_1	1141	405	200	↓(0.49×)	150	↓(0.37×)	85	↓(0.21×)

*Mean fluorescence determined by flow cytometric analysis.
†Change in mean fluoresence levels compared with the values obtained for MCF-7neo cells (indicated as ×) for the same integrin subunit.

some of the parameters involved in the apparent dynamics of interconversion.

In the development of the MoVi transfectants, it was obvious that each experimental clone was heterogeneous with respect to the actual percentage of vimentin IF-positive cells expressed. Furthermore, it is interesting to note that this percentage correlated well with the migratory ability of MoVi-10′ cells (65% vimentin-positive) and MoVi-9 (55%) *versus* MoVi-3 (47%). Indeed, further cloning selection of the MoVi-10 cells (originally containing 38% vimentin IF positivity) yielded the MoVi-10′ cells (expressing 65% vimentin IF positivity) and exhibiting an increase in invasive ability from 4.8 to 5.7% and in motility from 2.2 to 4.3%. The invasive and migratory potential of the MDA-MB-231 cells (100% vimentin IF positive) was significantly greater than the MoVi cells. These data suggest that, as the percentage of vimentin IF-positive cells (co-expressing with keratins 8 and 18) increased, so did invasive and migratory potential, especially when comparing the MoVi cells with the MDA-MB-231 cells. This observation coincides with a previous report of a mouse L cell model, which showed that transfectants co-expressing a low percentage of keratins 8 and 18 and vimentin IF-positive cells could be enriched *via* repeated migration through gelatin-coated polycarbonate membranes.[31] Thus, an increased percentage of L cells co-expressing both types of IFs correlate with the migratory ability of the cells.

Morphological assessment of keratins 8 and 18 and vimentin in MCF-7 parental cells, MCF-7neo sham transfectants, MoVi experimental transfectants, and MDA-MB-231 cells, using dual immunofluorescence labeling and confocal microscopy, showed remarkable IF networks. Although other studies have shown the expression of both keratins 8 and 18 and vimentin in MDA-MB-231 cells as well as transfected MCF-7 cells, only indirect and single labeling of these IFs was performed[6,7]; thus, no information could be obtained regarding the organization of diverse filament types within the same cell. Our results reveal apparently distinct keratin and vimentin IF networks, which vary somewhat in cellular distribution among the MoVi cells and the MDA-MB-231 cells. Whether the pattern of IF distribution is related to specific differences in biological function is as yet unknown. However, additional studies using the dual labeling procedure and confocal time-lapse videomicroscopy may assist in addressing this question. Indeed, other investigators have transfected keratins 8 and 18 expressing murine HR9 endoderm and F9 embryonic carcinoma cell lines with vimentin cDNA and have found a correlation between the amount of structural vimentin protein present and biological activity, such that aberrantly high levels can alter nuclear shape, retard cell spreading, and slow cellular growth.[32] Although our study did not focus on all the same parameters, with respect to proliferation, the MoVi-3 and MoVi-9 transfectants showed no significant change in proliferative capacity *in vitro* compared with the MCF-7neo cells. In fact, the vimentin-enhanced MoVi-10′ cells demonstrated a doubling time of 29 hours, which more closely resembles the MDA-MB-231 cell phenotype than the MCF-7 cells.

The most controversial aspect of this study involves the observation of the MoVi transfectants exhibiting increased invasive ability over the MCF-7neo transfectants and parental cells, in contrast to an earlier report by Sommers and colleagues.[7] In that study, MCF-7 cells were transfected with a full-length 1.8-kb human vimentin cDNA into the *Eco*RI site of pCMV-3 (a pUC19-derived plasmid containing the cytomegalovirus immediate-early region promoter), and the expression level of vimentin was determined by Western blot analysis simultaneous with the assessment of *in vitro* invasiveness through Matrigel-coated porous filters over 16 hours in response to chemoattractant. Regardless of the expression of vimentin protein, invasiveness was similar to the levels achieved by the MCF-7 controls. However, other MCF-7 cells tested in this study, which were selected for their resistance to adriamycin, demonstrated a constitutive level of expression of vimentin, in the form of basic protein and IFs, concomitant with an elevated invasive potential. Also, ZR-75-B breast cancer cells resistant to vinblastine turned on the expression of vimentin. Our study differs significantly in the experimental approach and measurement(s) of functional activity. For instance, the MCF-7 cells used in the present report were transfected with a full-length 1.8-kb *Bam*HI mouse cDNA, under the control of the human β-actin promoter. In addition, we correlated the presence of vimentin IFs with biological function, based on our previous observations of poorly to moderately invasive melanoma cells that co-expressed vimentin and keratins 8 and 18 proteins at the mRNA and protein levels but not in the form of IFs.[33] Lastly, we extended the observation window for invasion to 24 hours and used a human matrix barrier, composed of laminin/collagen IV/gelatin, that does not contain a myriad of hormones and growth factors that could introduce another experimental variable to interpret for these estrogen-dependent cells. Thus, we believe that the MoVi cells allowed additional opportunities to examine the potential significance of vimentin IF expression and

function under different, and possibly more optimal, experimental conditions. Indeed, an intriguing speculation regarding a potential role for IFs in cells co-expressing vimentin and keratins could be the conference of drug resistance. This has been demonstrated by vimentin-positive mouse L cells transfected with keratins 8 and 18 resulting in conference of a multiple drug resistance phenotype on cells exposed to mitoxantrone, doxorubicin, methotrexate, melphalan, colcemid, and vincristine[34] and, indirectly, with human breast cancer cells ZR-75-B resistant to vinblastine and MCF-7 cells resistant to adriamycin, which turned on the expression of vimentin.[7] Whether the conference of drug resistance in these select vimentin- and keratin-expressing cells is causative or associative is unknown but warrants further consideration.

Another experimental strategy was employed to test the significance of vimentin IFs using the MoVi-10′ transfectants and the MDA-MB-231 cells (which are known to constitutively express keratins 8 and 18 and vimentin IFs, in addition to being highly invasive and metastatic[6,7]). Using this model, we tested the hypothesis that down-regulation of vimentin expression would result in decreased motility. Accordingly, with the application of antisense oligonucleotides to vimentin, we demonstrated that vimentin expression could be transiently down-regulated, and concomitant assessment of migration potential indicated a 70% decrease in cell movement compared with controls in the MDA-MB-231 cells and a 50% decrease in migratory ability of the MoVi-10′ cells. Although this approach offers a narrow window for observation, it was sufficient in which to investigate potential changes in random cell migratory behavior and allowed a direct measurement of up-regulation and down-regulation of vimentin expression in the same cell line (MoVi-10′). Indeed, this experimental strategy has met with success in the study of epithelial-mesenchymal transformation in embryonic cells[35] as well as vimentin studies in neuritogenesis.[36]

One of the most important issues to address with the stable MoVi transfectants related to the clonogenic and tumorigenic potential, as a logical extension of the invasion and proliferation studies. The information derived from these studies indicated that MoVi-9 and MoVi-10′ cells formed colonies in soft agar, in fact, significantly more effectively than MoVi-3 and MCF-7neo cells. However, MDA-MB-231 cells formed the greatest number of colonies under these conditions. The tumorigenic data coincided with these findings, demonstrating larger primary tumors formed by MoVi-9 and MoVi-10′ in the mammary fat pads but not as large as the MDA-MB-231 cells. Of major significance was the finding that, during the 38-day incubation period in SCID mice, only the MDA-MB-231 cells were capable of forming metastases, which suggested that the addition of vimentin to MCF-7 cells was not sufficient to confer the metastatic phenotype inherent to MDA-MB-231 cells, at least not during this incubation period. In our experience, the SCID mouse model offers an accelerated window of observation for the metastasis of human tumor cells. Thus, although in highly invasive and metastatic breast cancer (human as well as mouse), co-expression of vimentin and keratins[6,7,37] have been shown to be associated with advanced disease, the simple experimental addition of vimentin to keratin-positive MCF-7 cells was not in itself sufficient to confer the complete metastatic phenotype. Although the regulation of vimentin expression in breast cancers is not fully understood, a recent investigation reported the absence of vimentin silencer protein binding activity in MDA-MB-231 cells and its presence in MCF-7 cells, thus providing a clue as to the transcriptional regulation of vimentin in these cells.[38] Additional studies in this area would be most appropriate.

Although the exact function of IFs, especially the co-expression of IFs in breast cancer, remains enigmatic, we have evidence from some of our previous studies with melanoma that suggests that the ability to co-express these IFs confers a selective advantage to tumor cells in their interpretation of signaling cues from mesenchymal and epithelial ECMs.[39] We were able to further test this speculation in the MoVi cells and found tremendous consistency with respect to the general trends in integrin shifts. Prevailing evidence demonstrating the importance of the ECM with regard to epithelial-mesenchymal transformation during embryological events and during mammary development and carcinogenesis[35,40,41] further prompted our focus on the integrin family of cell surface receptors.[42–47] We specifically measured integrins that would have the most involvement in the attachment of breast carcinoma cells to a laminin/collagen IV/gelatin matrix, as used in this study and found in basement membranes. During the interconversion of MCF-7 cells to a more invasive phenotype, demonstrated by the MoVi cells, we observed a decrease in the promiscuous α_2 and α_3 integrin subunits as well as the β_1-containing integrins, whereas an increase in the α_6 integrin subunit was seen, suggesting a potential increased affinity to laminin. However, when one compares the integrins measured on MDA-MB-231 cells with any of the MoVi cells or MCF-7neo controls, the integrin values of the MDA-MB-231 cells are much higher in every case.

This is not too surprising as the MDA-MB-231 cells are biologically quite different than the MCF-7 cells, especially with respect to their invasive and metastatic potential. To further investigate potential functional changes that might have accompanied shifts in integrin subunits, we measured the haptotactic migration potential of the MoVi cells toward laminin. These data showed a consistent increased response of these cells toward the laminin substrate compared with the MCF-7neo cells but no change in their ability to migrate toward the gelatin control substrate. Of course, the highly invasive and metastatic MDA-MB-231 cells exhibited a greater ability to migrate toward laminin, as well as gelatin, in addition to displaying higher levels of the selected integrin subunits on their surface. Indeed, laminin is a potent ECM signaling site that has diverse biological activities.[48] Although we do not have the complete analysis of the full integrin profile (with α and β components), it is reassuring to know that our results coincide with a recent report indicating that the re-expression of the $\alpha_2\beta_1$ integrin abrogates the malignant phenotype of breast carcinoma cells.[49] Indeed, our data show a decrease in these integrin subunits coincident with increased invasive behavior. Future studies involving the characterization of integrins in this model will provide a better understanding of the mechanisms involved in phenotypic interconversion. There is a growing body of evidence suggesting that transmembrane integrins are the most likely receptors to interact with IFs and their linking proteins,[50] mediating cell shape, spreading and migration by effecting changes in the cytoskeleton *via* a variety of signal transduction pathways.[45,51–53] Specifically, interactions between cell surface fibronectin and vimentin IFs have been observed[54] as well as contacts between α-actinin and the β_1 integrin subunit,[55] collectively suggesting the existence of a morphological transduction pathway.

Tumor cell phenotype is not autonomously determined by the tumor but, rather, reflects influences of the host environment.[56] The morphological and biological changes associated with the breast carcinoma interconverted phenotype suggest a direct link between keratin and vimentin IF co-expression, changes in integrin profile, and increased invasive, clonogenic, and tumorigenic activity. However, the expression of keratin or vimentin IFs alone is not sufficient to confer the complete metastatic phenotype. Thus, associated regulatory pathways need to be investigated with respect to the underlying molecular pathology of metastatic breast cancer to better understand the significance of IF co-expression in the interconverted tumor cell phenotype.

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